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Characterization of Xylanase from *Lentinus edodes* M290 Cultured on Waste Mushroom Logs

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Abstract Extracellular enzymes from *Lentinus edodes* M290 on normal woods (*Quercus mongolica*) and waste logs from oak mushroom production were comparatively investigated. Endoglucanase, cellobiohydrolase, β-glucosidase, and xylanase activities were higher on waste mushroom logs than on normal woods after *L. edodes* M290 inoculation. Xylanase activity was especially different, with a three times higher activity on waste mushroom logs. When the waste mushroom logs were used as a carbon source, a new 35 kDa protein appeared. After the purification, the optimal pH and temperature for xylanase activity were determined to be 4.0 and 50°C, respectively. More than 50% of the optimal xylanase activity was retained when the temperature was increased from 20 to 60°C, after a 240 min reaction. At 40°C, the xylanase maintained 93% of the optimal activity, after a 240 min reaction. The purified xylanase showed a very high homology to the xylanase family 10 from *Aspergillus terreus* by LC/MS-MS analysis. The highest Xcorr (1.737) was obtained from the peptide KWI SQGIPIDGIG SQTHLGSGGS WTVK originated from *Aspergillus terreus,* indicating that the 35 kDa protein was xylanase. This protein showed low homology to a previously reported *L. edodes* xylanase sequence.

Keywords: Xylanase, waste mushroom logs, *Lentinus edodes*, purification

Xylanases, mainly produced by microorganisms, hydrolyze the 1,4-β-D-xylosidic linkage of the main xylan chain to produce xylooligosaccharide. Xylan is the major component in plant hemicellulose [2, 23]. Fungal xylanases are especially interesting because these enzymes are secreted into liquid media, with activities much higher than those found in yeast or bacteria [15, 26]. Therefore, fungal xylanases have many commercial uses in the pulp and paper, animal feed, food, and drink industries [23]. Recently, xylanases have been used with cellulases to hydrolyze lignocellulosic biomass into simpler compounds for bioethanol production, to be used as alternative energy [3, 7]. Hemicellulose hydrolysis for bioethanol production from woody biomass is important, not only to recover monosaccharides from residual hemicellulose, but also to remove hemicellulose from hindering cellulase access to cellulose fibers. Berlin *et al.* [3] reported that the hardwood hydrolysis efficiency was increased by the presence of xylanase as an accessory enzyme. Additionally, xylanases were also used alone to produce pure cellulose preparations [2]. The applications of xylanases from various microorganisms have been increased by characterizing substrate properties, and pH and temperature stability [6, 8, 16, 20]. Many researchers have studied the optimum conditions for xylanase induction [22, 25].

Waste logs from oak mushroom production are considered suitable biomass for bioethanol production. The physical, chemical, and biological conversions are easy to perform because of the degradation of chemical components by enzymes secreted from *L. edodes* M290. Additionally, they have a low crystallinity value [13] and are easily made to induce cellulase or hemicellulase.

L. edodes, used to spawn oak mushrooms, has traditionally been grown on freshly cut *Corpinus* spp. and *Quercus mongolica* logs. The fungus produces hydrolytic and oxidative enzymes that degrade the woods, depending on substrate composition and environmental conditions [11, 22, 25]. Many researchers have reported on the *L. edodes* lignin degradation enzymes, manganese peroxidase and laccase, although lignin peroxidase apparently does not play a significant

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role in the ligninolytic enzymatic system [5, 21]. Additionally, *L. edodes* secretes exo-β-1,3-glucanase, β-glucosidase, cellobiohydrolase, and endoglucanase as cellulose degradation enzymes, and xylanase as hemicellulase [18, 19, 21, 24]. Therefore, waste logs from mushroom production are considered a suitable substrate for cellulases and hemicellulases [9].

In this study, activities of enzymes secreted from *L. edodes* M290 on normal woods and waste mushroom logs were investigated during cultivation. Among the enzymes, the induced xylanase with high activity on waste mushroom logs was characterized.

MATERIALS AND METHODS

Preparation of Lignocellulosic Biomass

Quercus mongolica (normal woods) and waste logs at least three years after oak mushroom cultivation were obtained from a mushroom farm located at *Hwasung-si, Gyeonggi-do,* Korea. Waste mushroom logs were washed with sterile distilled water to remove surface mycelium and dried to less than 10% moisture content. Both the normal woods and waste mushroom logs were milled to 40-mesh wood powder using a milling machine.

Microorganisms

Lentinus edodes M290 was spawned for oak mushroom production. The culture was provided by the National Forestry Cooperative Federation in Korea. The fungus was maintained on potato dextrose agar (PDA) medium at 28°C for 14 days, and stored at 4°C until further processing.

Culture Conditions

L. edodes M290 was cultivated on Kremer and Wood medium to assay cellulase and hemicellulase activities on different carbon sources. The medium was composed of 2.6 g/l $(NH_4)_2HPO_4$, 1.1 g/l KH_2PO_4, 2.2 g/l dimethylsuccinic acid, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 1 ml Trace 1 ($CaCl_2 \cdot 2H_2O$ 740 mg, $CoCl_2 \cdot 6H_2O$ 10 mg, per liter) and 1 ml Trace 2 ($FeSO_4 \cdot 7H_2O$ 100 mg, $MnSO_4 \cdot 7H_2O$ 50 mg, $ZnSO_4 \cdot 7H_2O$ 50 mg, per liter) [14]. To prepare the culture, 10 plugs (8-mm diameter) of mycelium were punched out and used to inoculate 200 ml of liquid medium in a 500-ml Erlenmeyer flask. The cultures were incubated at 28°C for 30 days.

Enzyme Assays and Protein Determination

Endo-1,4-β-glucanase (EG) activity was assayed using ostazin brilliant red-hydroxyl cellulose (OBR; Sigma Chemical Co., U.S.A.). The reaction mixture (750 μl) contained 25 μl of 2.5 mg OBR/ml and 10 μl enzyme solution in 50 mM citrate-phosphate buffer (pH 4.8). After incubating for 15 min at 40°C, the reaction was terminated by adding three volumes of ethanol/acetone (2:1, v/v). Release of the chromogenic product from the polymeric substrate was monitored by increased absorbance at 550 nm [27].

Cellobiohydrogenase (CBH) activity on a chromogenic substrate was measured as follows. The reaction mixture consisted of 0.4 ml of 5 mM *p*-nitrophenyl β-D-lactoside (pNPL; Sigma Chemical Co., U.S.A.), 0.4 ml of enzyme solution, and 0.8 ml of 50 mM sodium acetate buffer (pH 5.0). The mixture was incubated at 40°C for 60 min, and 2.0 ml of 1% (w/v) sodium carbonate solution was added to the reaction. The *p*-nitrophenol released from the substrate was measured colorimetrically at 420 nm [10].

β-Glucosidase (BGL) activity was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma Chemical Co., U.S.A.). The enzyme solution (100 μl) was incubated for 5 min at 30°C with 1 mM *p*-nitrophenyl-β-D-glucopyranoside in 10 mM sodium acetate buffer (pH 5.0). The enzymatic reaction was stopped by adding 100 μl of 2 M sodium carbonate. The *p*-nitrophenol released by β-glucosidase was quantitated as described above [12].

Xylanase activity was determined using RBB-xylan (Sigma Chemical Co., U.S.A.) as a substrate. Fifty μl of RBB-xylan solution (10 mg/ml) was added to 100 μl of enzyme solution and 150 μl of 50 mM sodium acetate buffer (pH 4.5). After incubating for 30 min at 30°C, the reaction was stopped by adding two volumes of 95% ethanol. The samples were spun, and the OD of the supernatant, containing the soluble digested xylan, was measured at 620 nm [19].

Protein concentrations were determined by the Bradford method, using 1 mg/ml bovine serum albumin (BSA; Sigma Chemical Co., U.S.A.) as a standard [4]. The reaction mixture contained 1 ml of Bradford reagent and 0.1 ml of sample solution. The absorbance was measured at 595 nm within 1 h.

Enzyme Purification

L. edodes M290 cultures grown on Kremer and Wood medium were spun down (30 min, 10,000 ×*g*) and the supernatant was incubated with 5% (w/v) bentonite for 30 min at room temperature. After the bentonite was removed by centrifugation, the solution was concentrated with a PY-10 ultrafiltration membrane (Amicon, U.S.A.). The concentrated sample was purified on an FPLC system (Amersham Pharmacia Biotech, AKTA Explorer 10). The sample was put on a Mono Q ion-exchange column (Amersham Pharmacia Biotech, 0.5×5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0). Bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate 0.5 ml/min. The active fractions were flowed over a Superdex 75 size exclusion column (Amersham Pharmacia Biotech, 100× 300 GL) equilibrated with 20 mM sodium acetate (pH 5.0). The fractions containing xylanase activity were collected, concentrated, and used for further study. All purification

steps were performed at 4°C. To determine protein molecular masses, SDS-PAGE was done according to the procedure of Laemmli on 12% polyacrylamide gel [17].

Optimal pH and Temperature for Xylanase Activity

To determine the optimal pH, xylanase activity was measured at various pHs in different buffer solutions (glycine, pH 2.5–3.5; sodium acetate; pH 4.0–5.5; sodium phosphate, pH 6.0–8.0). The optimal temperature was estimated at temperatures between 20 and 60°C. The enzyme activity was determined by the standard enzyme reaction. To determine thermostability, the enzyme was incubated in 50 mM sodium acetate buffer (pH 4.5) at different temperatures for various reaction times. After cooling the enzyme, xylanase activity was measured.

Identification of Enzyme by LC/MS/MS

After an in-gel digestion [1] of a protein band, the sample was injected to a column made with a Surveyor autosampler (Surveyor; Thermo Finnigan, San Jose, CA, U.S.A.). A fused silica capillary microcolumn (254 μm i.d., 358 μm o.d., Polymicro Tech., Phoenix, AZ, U.S.A.) was prepared with a P-2000 laser puller (Sutter Instruments, Novato, CA, U.S.A.). The 10-cm-long microcolumn was packed with 5-μm C18 resin (200 Å, Phenomenex, U.S.A.) using a pressure bomb. The peptide separation consisted of several steps: loading with buffer A [20% (v/v) ACN/0.1% formic acid] for 10 min, a linear gradient from 5 to 30% buffer B [80% (v/v) ACN/0.1% formic acid] for 40 min, a linear gradient from 30 to 50% buffer B for 15 min, a linear gradient from 50 to 80% buffer B for 10 min, a linear gradient from 80 to 5% buffer B for 5 min, and 5% buffer B for 20 min. The eluent from the column transferred directly into the electrospray ionization source of a Thermo Finnigan LCQ DecaXPplus ion-trap mass spectrometer. Automated peak recognition, dynamic exclusion, and daughter ion scanning of the two most intense ions were performed with the XCALIBUR software. The SEQUEST algorithm was used to interpret the MS/MS data. Database information was downloaded from ExPaSy (expert protein analysis system, http://kr.expasy.org/).

RESULTS

Enzyme Activities During *L. edodes* M290 Cultivation

Differences in enzyme activities from *L. edodes* M290 in submerged culture on normal woods and waste mushroom logs for 30 days are presented in Fig. 1. Cellobiohydrolase and β-glucosidase activities were relatively lower than endoglucanase and xylanase. All cellulose degradation enzymes had higher activities on waste mushroom logs

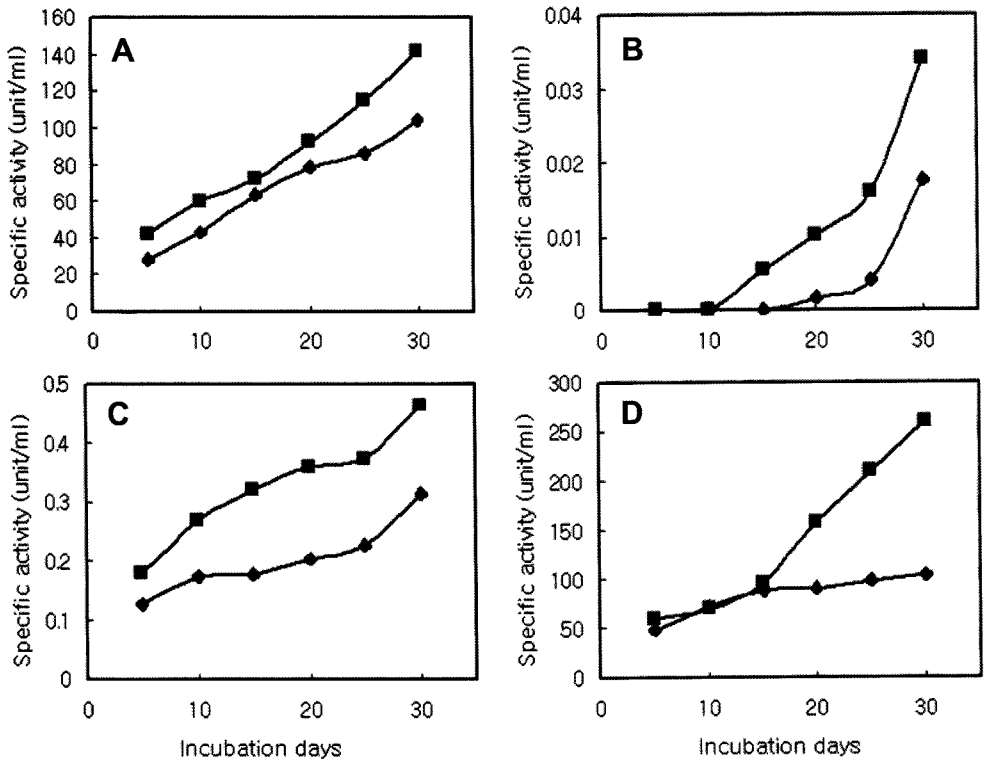

Fig. 1. Differences in enzyme activities on normal woods (◆) and waste mushroom logs (■) during cultivation of *L. edodes* M290.
A. Endoglucanase; **B**. Cellobiohydrolase; **C**. β-Glucosidase; **D**. Xylanase.

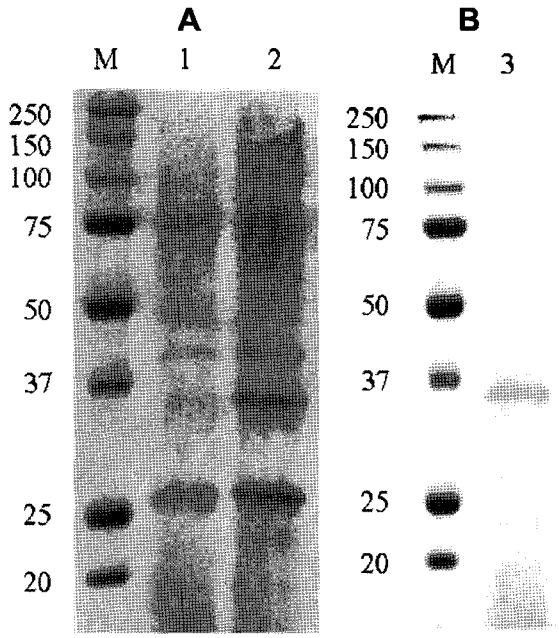

Fig. 2. Protein pattern of extracellular enzymes from *L. edodes* M290 on normal woods and waste mushroom logs.
A. Crude enzymes; **B**. Purified enzyme. M: Molecular mass standard; lane 1: normal woods; lane 2: waste mushroom logs; lane 3: purified xylanase.

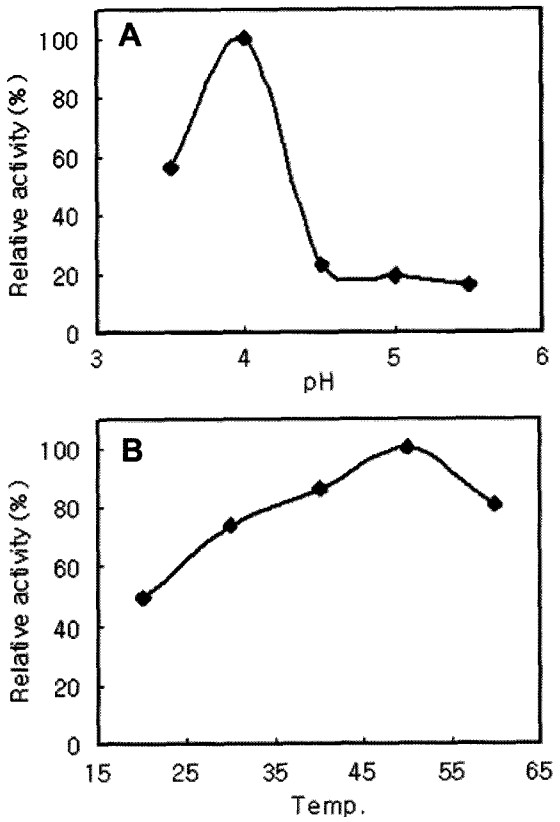

Fig. 3. Effect of pH (**A**) and temperature (**B**) on xylanase activity from *L. edodes* M 290.

than on normal woods. Xylanase, a typical hemicellulase, was three times higher on waste mushroom logs than on normal woods. Xylanase activity dramatically increased after 15 days on waste mushroom logs. These findings agree with previous data showing that *L. edodes* xylanase was activated by forestry waste and wheat straw [22, 25]. High cellulase and hemicellulase activities from *L. edodes* M290 on waste mushroom logs could more easily degrade monosaccharides or oligosaccharides. We believe the increased activity is due to the decrease of waste mushroom log crystallinity, a result of secreted enzymes from spawns during mushroom growth. Therefore, a low crystallinity value could allow for increasing cellulase and hemicellulase accessibility. The relatively higher xylanase activity suggests that waste mushroom logs are a good substrate for xylanase production.

Xylanase Purification

The comparison of extracellular protein patterns from *L. edodes* M290 cultured on normal woods and waste mushroom logs are shown in Fig. 2A. The protein patterns from the two carbon sources were very similar except for a difference near 35 kDa. Many researchers have reported on extracellular enzymes from *L. edodes* cultured on various carbon sources. A 53 kDa band was previously shown to be endoglucanase [18]. A band near 28 kDa corresponded to the 283-amino acid, 29.5 kDa xylanase, glycoside hydrolase 11 from *L. edodes* [19]. Based on the xylanase activity on waste mushroom logs, the 35 kDa protein could be xylanase.

To characterize the xylanase, purification and identification were carried out. After xylanase purification using ultrafiltration and two consecutive column chromatographies, SDS-PAGE revealed a single band with a molecular mass of about 35 kDa. The single purified xylanase band is shown in

Table 1. Purification of xylanase from *L. edodes* M290 cultured on waste mushroom logs.

Purification step	Total volume (ml)	Total protein (mg)	Volume activity (U/ml)	Total activity (U)	Specific activity (U/mg)
Crude culture	1,000	49.35	0.269	269	5.45
Ultrafiltration	50	11.2	3.649	182.45	16.29
Mono Q	20	1.04	1.565	31.3	30.1
Superdex 75	40	0.47	1.062	42.48	90.38

Fig. 2B. Xylanase activity from the *L. edodes* M290 supernatant at each purification step is summarized in Table 1. The activity yield of the first step was 67.83%. The pooled xylanase fractions were concentrated before the second step. The final enzyme was purified to 16.58-folds with a specific activity of 90.38 U/mg, compared with 5.45 U/mg at the crude culture step and a yield of about 15.79%.

Optimal pH and Temperature for Xylanase Activity

Xylanase activities at varying pH and temperatures are shown in Fig. 3. The optimal pH was 4.0, which corresponds with data from Lee *et al.* [19]. The activity rapidly decreased with increasing pH. The optimum pH was a little different than the reported optimum of pH 4.5 [19]. Xylanase activity increased at temperatures between 20 and 50°C, and slightly decreased at those over 50°C. At various temperatures, the relative activity was retained over 50%. Therefore, purified xylanase from *L. edodes* M290 can be considered a novel xylanase with unique characteristics, compared with the xylanase reported by Lee *et al.* [19].

The thermostability profile of purified xylanase from *L. edodes* M290 at 40, 50, and 60°C is presented in Fig. 4. The thermostability assay showed that purified xylanase was highly stable at 40°C. Approximately 93% of xylanase activity was maintained when incubated at 40°C for

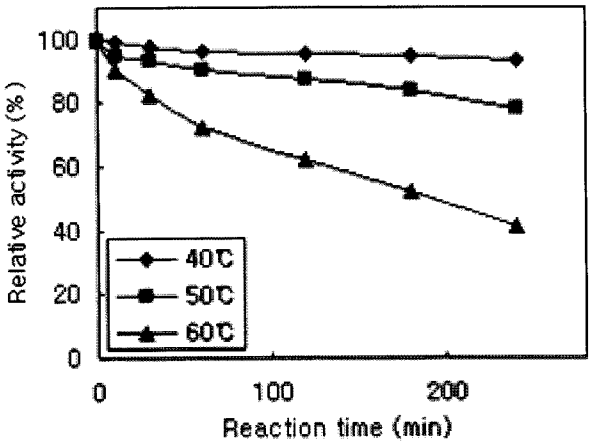

Fig. 4. Thermostability of xylanase purified from *L. edodes* M290.
◆, 40°C; ■, 50°C; ▲, 60°C.

240 min. At the same time, xylanase retained 78.12% and 41.64% of the maximum activity at 50 and 60°C, respectively. At temperatures up to 60°C, xylanase activity decreased sharply with time (data not shown).

Identification of Xylanase

To identify the single band near 35 kDa on SDS-PAGE, the band was separated and purified. Peptide finger-

Table 2. Result of BLAST homology search of amino acid sequences from a 35 kDa band secreted from *L. edodes* M290.

Scan (s)	Reference	MH+	z	Peptide	P (pep)	XC	DeltaCn	Sp	RSp	Ions
2078	AAY86996 xylanase family 10 [*Aspergillus terreus*]	2809.46315	3	R.KWISQGIPIDGIGSQ THLGSGGSWTVK.D	2.03E-01	1.737	-	252.3	1	18/104
1874	AAY86996 xylanase family 10 [*Aspergillus terreus*]	1575.84241	2	K.NHITTVMQRYKGK.V	1.06E-01	1.321	0.875	273.6	1	38649
2045	AAY86996 xylanase family 10 [*Aspergillus terreus*]	1803.85480	3	K.LYINDYNLDNANYA K.T	1.40E-02	1.206	-	120.0	1	10/56
1940	CAA31109 xylanase [*Filobasidium floriforme*]	1868.92448	2	K.MKLCINDYNIETVNA K.S	4.80E-02	1.079	-	39.4	1	38563
4647	AAY86996 xylanase family 10 [*Aspergillus terreus*]	2407.09830	3	K.ADFGQLTPENSMKW DATEPNR.G	2.30E-01	1.066	-	86.8	1	16/80
2159	CAA31109 xylanase [*Filobasidium floriforme*]	1868.92448	2	K.MKLCINDYNIETVNA K.S	5.68E-01	1.027	-	39.3	1	38502
1421	AAY86996 xylanase family 10 [*Aspergillus terreus*]	1099.56769	2	K.NHITTVMQR.Y	1.05E-01	1.018	-	142.3	1	38519
2108	CAA31109 xylanase [*Filobasidium floriforme*]	1868.92448	2	K.MKLCINDYNIETVNA K.S	6.64E-01	1.016	-	73.8	1	38563

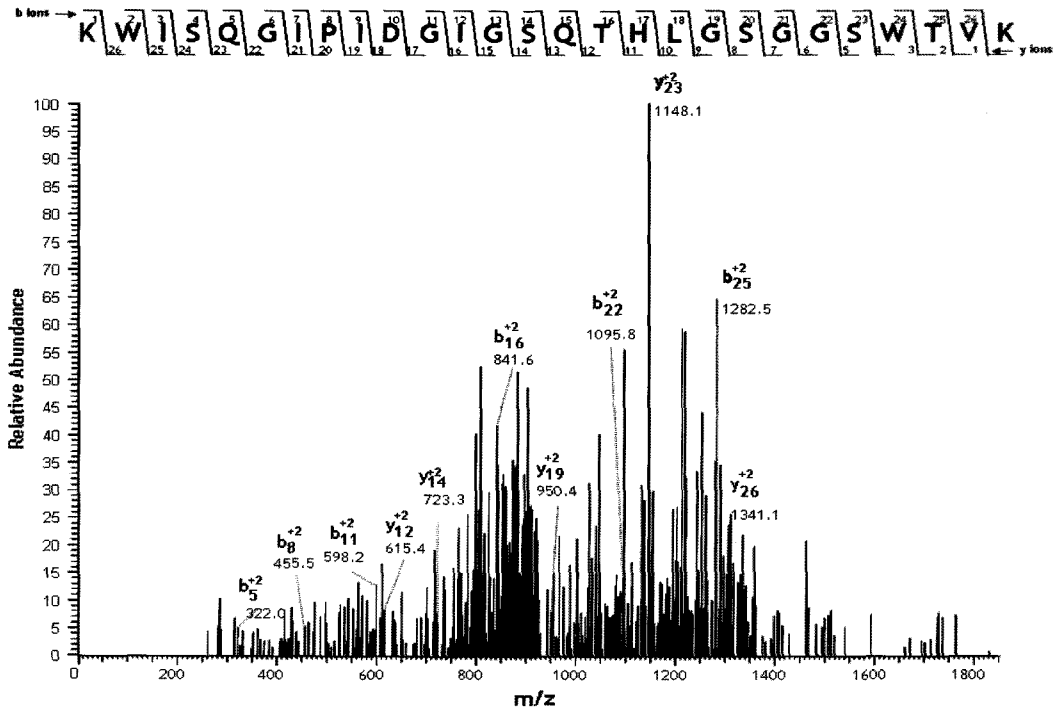

Fig. 5. Collision-induced dissociation mass spectrum m $[M+2H]^{2+}$ ions at m/z 936.64 of the amino acid sequence KWI SQGIPIDGIG SQTHLGSGGS WTVK produced by trypsin treatment.

printing indicated a high homology to xylanase. The amino acid sequence obtained from the mass peaks was compared with the NCBI xylanase sequence. The 35 kDa protein showed the highest homology to the xylanase family 10 from *Aspergillus terreus*. The results of the BLAST homology search are shown in Table 2. The peptides KADFGQLTP ENSMKWDATE PNR, KNHITTV MQRYKGK, KLYINDYN LDNANYAK and KWI SQGIPIDGIG SQTHLGSGGS WTVK corresponded to xylanase family 10 from *A. terreus* with Xcorr values of 1.066, 1.321, 1.206, and 1.737, respectively. KWI SQGIPIDGIG SQTHLGSGGS WTVK had the highest Xcorr value, as presented in Fig. 5. KMKLCINDYN IETVNAK corresponded with an induced xylanase from *Filobasidium floriforme* with an Xcorr value of 1.079. This peptide also showed low homology to glycoside hydrolase 11, as reported by Lee *et al.* [19]. The induced xylanase from *L. edodes* M290 cultured on waste mushroom logs is considered a novel xylanase.

Discussion

Endoglucanase and xylanase, which degrade cellulose and hemicellulose, had higher activities than did β-glucosidase and cellobiohydrolase on normal woods. On waste mushroom logs, the pattern of secreted enzymes is similar to that on normal woods. Xylanase activity on waste mushroom logs is three times higher than that on normal woods. Thus, waste mushroom logs may be a good substrate for xylanase induction. Following identification, the xylanase has high homology with a fungal xylanase, and a low homology with a previously reported xylanase from *L. edodes*. Therefore, molecular biology work and kinetics regarding the substrate for the new xylanase must be performed.

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